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Molecular and Phenotypic Analyses Reveal Association of Diverse *Colletotrichum acutatum* Groups and a Low Level of *C. gloeosporioides* with Olive Anthracnose

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Anthracnose (*Colletotrichum* spp.) is an important disease causing major yield losses and poor oil quality in olives. The objectives were to determine the diversity and distribution pattern of *Colletotrichum* spp. populations prevalent in olives and their relatedness to anthracnose pathogens in other hosts, assess their pathogenic variability and host preference, and develop diagnostic tools. A total of 128 *Colletotrichum* spp. isolates representing all olive-growing areas in Portugal and a few isolates from other countries were characterized by molecular and phenotypic assays and compared with reference isolates. Arbitrarily primed PCR data, internal transcribed spacer of rRNA gene and β-tubulin 2 nucleotide sequences, colony characteristics, and benomyl sensitivity showed *Colletotrichum acutatum* to be dominant (>97%) with limited occurrence of *Colletotrichum gloeosporioides* (<3%). Among *C. acutatum* populations, five molecular groups, A2 to A6, were identified. A2 was widely prevalent (89%), coinciding with a high incidence of anthracnose and environmental conditions suitable to disease spread. A4 was dominant in a particular region, while other *C. acutatum* groups and *C. gloeosporioides* were sporadic in their occurrence, mostly related to marginal areas of olive cultivation. *C. gloeosporioides*, isolated from olive fruits with symptoms indistinguishable from those of *C. acutatum*, showed same virulence rating as the most virulent *C. acutatum* isolate from group A2. *C. acutatum* and *C. gloeosporioides* isolates tested in infected strawberry fruits and strawberry and lupin plants revealed their cross-infection potential. Diagnostic tools were developed from β-tubulin 2 sequences to enable rapid and reliable pathogen detection and differentiation of *C. acutatum* groups.

Several species belonging to the genus *Colletotrichum* cause important diseases in a wide range of crops including cereals, legumes, vegetables, and fruit crops. Olive (*Olea europaea* subsp. *europaea*) is an important crop in the Mediterranean basin, and olive anthracnose is becoming an increasingly important disease in this region. The disease is very common in Portugal, causing up to 100% losses, particularly in the widely cultivated variety Galega, but the disease is also spreading in other Mediterranean countries such as Spain (22), Italy (3), and Serbia and Montenegro (38). Symptoms typically occur on fruits at maturation under wet autumn conditions, as dark sunken lesions with abundant production of orange masses of conidia. Symptoms on stems and leaves are only rarely observed. The disease causes premature fruit drop, poor oil quality (high acidity), and damaged fruits unacceptable for canning. The olive anthracnose pathogen was originally described as *Gloeosporium olivarum* Alm. but was later transferred to *Colletotrichum gloeosporioides* (Penzig) Penzig & Saccardo (37). More recently, based on a limited collection of isolates, both *Colletotrichum acutatum* Simmonds ex Simmonds and *C. gloeosporioides* were reported to be associated with the disease (21). However, very little information is available on the relative importance of each of these species and their diversity and distribution. In anthracnose pathosystems, a number of examples are known where more than one *Colletotrichum* species is associated with the same host, and a single *Colletotrichum* species is able to infect a range of hosts (10). For example, *C. acutatum* is associated with the postbloom fruit drop and anthracnose of different types of citrus, while *C. gloeosporioides* is a postharvest pathogen of citrus but also occurs commonly as a saprophyte (4). So it is critical to be able to accurately diagnose the pathogen and understand its epidemiology to develop effective disease management. Moreover, differential sensitivity of these mixed *Colletotrichum* populations to fungicides such as benomyl can pose problems in disease control, as well as lead to shifts in pathogen populations (11). Molecular markers in conjunction with phenotypic analyses have been successfully applied to address these issues in various anthracnose pathosystems (2, 7, 34).

The objectives of this work were (i) genotypic and phenotypic characterization of *Colletotrichum* spp. isolates associated with olive anthracnose to identify the key pathogen(s), (ii) to determine the extent of diversity and distribution of the pathogen(s), as well as their relatedness to *Colletotrichum* spp. populations from other hosts, (iii) to assess the pathogenic variability and cross-infection potential of these groups, and (iv) to develop tools for pathogen detection, as well as diagnosis of subspecific molecular groups, to facilitate improved disease management.

MATERIALS AND METHODS

Fungal isolates. A total of 128 *Colletotrichum* spp. isolates were established from olive anthracnose samples collected during autumns (October to December) of 2001, 2002, and 2003 from different olive production areas in Portugal

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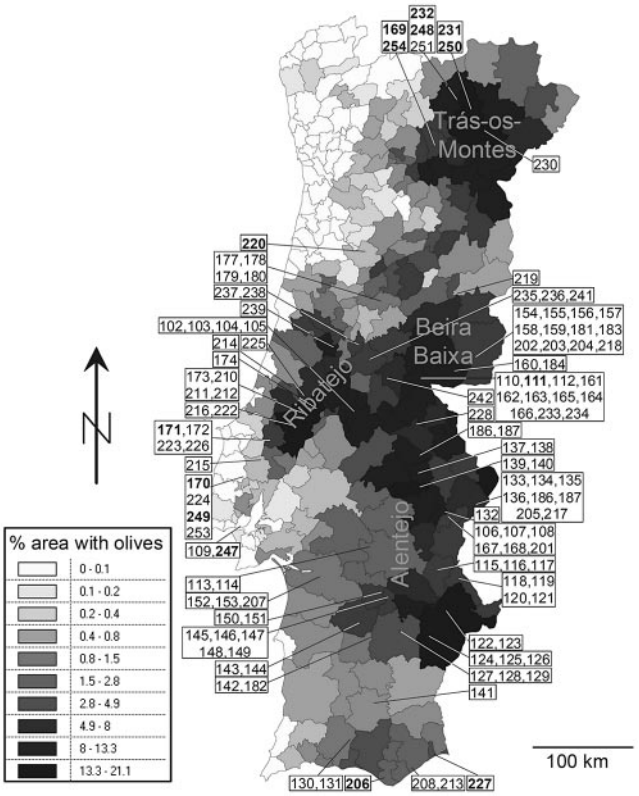

FIG. 1. Map of Portugal depicting percentage of olive areas per municipality, main olive-growing areas (Alentejo, Ribatejo, Beira Baixa, and Trás-os-Montes) and origin of *Colletotrichum* spp. isolates. For isolates collected from different locations within a municipality, a line points to the headquarters of the municipality. Numbers in boldface type represent isolates that do not belong to *C. acutatum* group A2, which are PT111, VM206, and PR220, *C. gloeosporioides*; PT170 and PT249, *C. acutatum* group A3; PT169, PT171, PT231, PT232, PT247, PT248, and PT254, *C. acutatum* group A4; PT227, *C. acutatum* group A5; and PT250, *C. acutatum* group A6. Isolate details are given in Table 1.

(Fig. 1 and Table 1). In a number of cases, more than one isolate was obtained from each location/sample, to assess the presence of mixed populations and to monitor any shifts in pathogen distribution related to disease incidence/severity. These isolates are identified in Table 1 under the column "Location" by the letter A (analysis for mixed populations) or letters B to H (pathogen and disease monitoring of locations and surrounding sites in different years). Other olive anthracnose isolates analyzed were CBS193.32, collected in Italy in 1932, and M3 and M4, collected in Serbia and Montenegro in 2003, which were all supplied as *C. gloeosporioides*. Previously characterized (31, 34) *C. acutatum* isolates PD88/673 (isolated from *Anemone* sp.); JG05 (*Ceanothus* sp.); PD85/694 (*Chrysanthemum* sp.); CMG12 (*Cinnamomum zeylanicum*); PD89/582 (*Cyclamen* sp.); TN47 (*Eriobotrya japonica*); C2897, CR20, NI90, and CA397 (*Fragaria*× *ananassa*); CA473 (*Liriodendron tulipifera*); HO01, JR03, HY09, and PT30 (*Lupinus albus*); CA318 (*Magnolia* sp.), CA302a (*Nandina domestica*); PD443 (*Phlox* sp.); CA455 (*Photinia* sp.); CA287 (*Statice* sp.); CR46 (*Vitis vinifera*); and *C. gloeosporioides* isolates CR21 (*Citrus limon*), CR45 (*Citrus* sp.), and CG315 (*Fragaria*× *ananassa*) were used as references for comparative purposes. All isolates used in this study were monoconidial cultures and are stored as agar plugs in water at room temperature at the authors' laboratories. Nucleotide sequences (for the internal transcribed spacer of the rRNA gene [rRNA gene-ITS]) of a few other isolates retrieved from databases were also used as references in DNA sequence analysis and are referred to in the appropriate results section.

Assessment of morphological and cultural characteristics. For each isolate, the length and width of 30 conidia were measured, the length/width ratio was determined, and the shape was recorded. This was done with conidia obtained from cultures grown on potato dextrose agar (PDA; Difco, Detroit, MI), as well as Spezieller Nährstoffarmer Agar SNA (25). Data were analyzed using analysis of variance and the Tukey honest significant difference test with Statistica software (StatSoft, Tulsa, OK). The colony radius (three replicates; four measurements per replicate with statistical analysis as above) and characteristics (texture, density, color (29), zonation, transparency aspect, nature of the growing margin, presence of conidial masses, and color of the reverse side) were recorded from cultures grown for 5 days at 25°C under darkness on PDA plates. Benomyl sensitivity of the isolates was assessed by comparing colony radius on PDA and PDA amended with 2 mg · dm^{-3} benomyl (Benlate; Dupont, Wilmington, Delaware).

Pathogenicity assays. Pathogenicity tests were performed with a representative set of isolates using fruits of 11 different olive cultivars and 'Camarosa' strawberry. Fruits were surface disinfected by immersion for 30 s in 1% NaClO, followed by rinsing in sterile distilled water and inoculation by deposition of a 20-μl droplet of a conidial suspension of 10^5 conidia · cm^{-3} containing 1% gelatin on the fruit surface. The inoculated fruits, along with appropriate controls, were incubated in 100% relative humidity at room temperature (ca. 21°C), and symptoms were recorded after 7 days for strawberries and 11 days for olives. Lupin (*Lupinus albus* 'Rio Maior') and strawberry (*Fragaria* × *ananassa* 'Camarosa') plants were inoculated by spraying the conidial suspension, and symptoms were recorded after 11 days. Preparation of conidial suspension and postinoculation conditions were as described above.

Molecular analyses. For DNA extraction, fungal isolates were grown in petri dishes containing yeast extract (0.2% [wt/vol]) and glucose (1.0% [wt/vol]) liquid medium (both from Difco) for 4 days at 25°C. DNA was extracted from freeze-dried mycelium using the DNeasy Plant kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Arbitrarily primed PCR (AP-PCR) profiles were generated for each isolate using seven different primers (CAC_5, CAG_5, GAC_5, $GACG_4$, GCA_5, TCC_5, and MR [5′ GAGGGTGGCGGTTCT 3′]) and the composite data for each isolate were analyzed as previously described (34). Amplification and nucleotide sequencing of the rRNA gene-ITS region and a variable region of the β-tubulin 2 (*tub2*) gene were done as previously described (34).

PCR based detection of pathogens and diagnosis of molecular groups within *C. acutatum*. PCR primers TBCA (5′ CGGAGGCCTGGTTGGGTGAG 3′) specific for *C. acutatum* and TBCG (5′ CGGAAGCCTGGGTAGGAGCG 3′) specific for *C. gloesoporoides* were designed from the *tub2* sequences generated. Each of these primers was used in conjunction with the conserved primer TB5 (34) for diagnostic PCR of *C. acutatum* and *C. gloesoporoides* isolates or infected olive samples. Similarly, primers CaInt2 specific for *C. acutatum* (32) and CgInt specific for *C. gloeosporioides* (23) were each used in conjunction with the conserved primer ITS4 (39) for diagnostic PCR based on the rRNA gene-ITS region. Each PCR (25 μl) contained 25 ng DNA, 1 μM each primer, and 12.5 μl of ReadyMix RedTaq (Sigma-Aldrich, Gillingham, United Kingdom). Amplifications were performed with a thermal cycler (Proteus II; Helena Biosciences, Sunderland, United Kingdom) programmed for 1 cycle of 5 min at 95°C; 25 cycles, each of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C; and ending with 1 cycle of 7 min at 72°C. PCR products (each, 10 μl) were visualized by electrophoresis on 2% (wt/vol) agarose gels (Invitrogen, Paisley, United Kingdom). For the diagnosis of molecular groups within *C. acutatum* based on unique restriction fragment profiles, the *tub2* fragment amplified with primers TB5 and TBCA (specific for *C. acutatum*) and TB5 and TB6 (conserved) (34) were restriction digested using enzymes StyI, NlaIII, RsaI (New England Biolabs, Hitchin, United Kingdom), and SacI (Roche Diagnostics, Mannheim, Germany). All restriction reactions were performed with ca. 500 ng DNA for 2 h at 37°C using 4 U of enzyme, following manufacturers' instructions. Restriction fragments were separated by electrophoresis on 3% agarose gels.

Nucleotide sequence accession numbers. Nucleotide sequences generated were deposited in the EMBL database, and the accession numbers are shown in Table 1, footnote *a*. Nucleotide sequence analyses data were deposited in TreeBase with reference number SN2087.

RESULTS

AP-PCR analysis. Of the 131 *Colletotrichum* spp. isolates analyzed from olive anthracnose using AP-PCR markers (6.4 bands per isolate and per primer and an average of 21.1 distinct bands for all isolates per primer), 128 isolates grouped with various *C. acutatum* reference isolates, while 3 isolates grouped with the *C. gloeosporioides* reference isolates. Al-

TABLE 1. Main collection data for *Colletotrichum* spp. isolates from olive anthracnose samples used in this study, their identification, subspecific molecular grouping, and colony characteristics

Reference[a]	Location[b]	Collection date (mo-yr)	Grove, olive trees[c]	Topography[d]	Species[e]	*C. acutatum* groups	Colony diam after 5 days on PDA 25°C (mm)	Growth on PDA with benomyl (2 mg/dm^{-3}), 5 days at 25°C[f]
PT102	Qta. Pouchão, Abrantes (A)	Nov-01	6	2	CA	A2	38.5	1
PT103	Qta. Pouchão, Abrantes (A)	Nov-01	6	2	CA	A2	38.2	1
PT104	Qta. Pouchão, Abrantes (A)	Nov-01	6	2	CA	A2	41.3	1
PT105	Qta. Pouchão, Abrantes (A)	Nov-01	6	2	CA	A2	39.0	1
PT106	Vila Viçosa (A)	Nov-01	6	2	CA	A2	36.7	1
PT107	Vila Viçosa (A)	Nov-01	6	2	CA	A2	42.7	1
PT108	Vila Viçosa	Nov-01	6	2	CA	A2	36.5	1
PF109	Lisbon	Nov-01	2	2	CA	A2	34.8	1
PT110	Vila Velha de Ródão	Nov-01	6	3	CA	A2	38.8	1
PT111	Carapetosa, Vila Velha de Ródão (B)	Nov-01	4	3	CG		52.5	0
PT112	Vila Velha de Ródão	Nov-01	6	3	CA	A2	40.5	1
PT113	S. Sebastião da Giesteira, Évora (A)	Nov-01	6	2	CA	A2	39.3	1
PT114	S. Sebastião da Giesteira, Évora (A)	Nov-01	6	2	CA	A2	41.2	1
PT115	Caridade, Reguengos de Monsaraz (A)	Nov-01	6	2	CA	A2	41.8	1
PT116	Caridade, Reguengos de Monsaraz (A)	Nov-01	6	2	CA	A2	40.0	1
PT117	Caridade, Reguengos de Monsaraz (A)	Nov-01	6	2	CA	A2	37.5	1
PT118	Mourão (A)	Nov-01	6	1	CA	A2	37.3	1
PT119	Mourão (A)	Nov-01	6	1	CA	A2	42.7	1
PT120	Mourão (A)	Nov-01	6	1	CA	A2	39.5	1
PT121	Mourão (A)	Nov-01	6	1	CA	A2	40.8	1
PT122	Moura (A)	Nov-01	6	2	CA	A2	43.0	1
PT123	Moura (A)	Nov-01	6	2	CA	A2	37.3	1
PT124	Serpa (A)	Nov-01	6	2	CA	A2	45.5	1
PT125	Serpa (A)	Nov-01	6	2	CA	A2	43.3	1
PT126	Serpa (A)	Nov-01	6	2	CA	A2	38.7	1
PT127	Baleizão, Beja (A)	Nov-01	6	2	CA	A2	37.7	1
PT128	Baleizão, Beja (A)	Nov-01	6	2	CA	A2	40.8	1
PT129	Beja	Nov-01	6	1	CA	A2	40.5	1
PT130	Portela de Messines, Silves (A)	Nov-01	6	3	CA	A2	45.7	1
PT131	Portela de Messines, Silves (A)	Nov-01	6	3	CA	A2	46.2	1
PT132	Borba	Dec-01	5	2	CA	A2	41.3	1
PT133	Calçadinha, Elvas (A)	Dec-01	6	1	CA	A2	46.5	1
PT134	Calçadinha, Elvas (A)	Dec-01	6	1	CA	A2	44.7	1
PT135	Elvas (A,C)	Dec-01	6	2	CA	A2	36.2	1
PT136	Elvas (A,C)	Dec-01	6	2	CA	A2	39.3	1
PT137	Fronteira (A)	Dec-01	6	2	CA	A2	41.7	1
PT138	Fronteira (A)	Dec-01	6	2	CA	A2	37.7	1
PT139	Sousel (A)	Dec-01	6	3	CA	A2	40.7	1
PT140	Sousel (A)	Dec-01	6	3	CA	A2	38.8	1
PT141	Almodôvar	Nov-02	6	2	CA	A2	41.5	1
PT142	Ervidel, Aljustrel	Nov-02	3	1	CA	A2	35.8	1
PT143	Peroguarda, Ferreira do Alentejo (A)	Nov-02	6	1	CA	A2	38.5	1
PT144	Peroguarda, Ferreira do Alentejo (A)	Nov-02	6	1	CA	A2	35.2	1
PT145	Faro do Alentejo, Cuba (A)	Nov-02	5	1	CA	A2	35.5	1
PT146	Faro do Alentejo, Cuba (A)	Nov-02	5	1	CA	A2	35.2	1
PT147	Vila Ruiva, Cuba (A)	Nov-02	5	1	CA	A2	40.0	1
PT148	Vila Ruiva, Cuba (A)	Nov-02	5	1	CA	A2	33.0	1
PT149	Vila Ruiva, Cuba (A)	Nov-02	5	1	CA	A2	34.0	1
PT150	Alvito (A)	Nov-02	5	2	CA	A2	35.8	1
PT151	Alvito (A)	Nov-02	5	2	CA	A2	36.2	1
PT152	Torrão, Alcácer do Sal (A)	Nov-02	5	1	CA	A2	35.5	1
PT153	Torrão, Alcácer do Sal (A)	Nov-02	5	1	CA	A2	37.0	1
PT154	Oledo, Idanha-a-Nova (A)	Dec-02	6	1	CA	A2	32.0	1
PT155	Oledo, Idanha-a-Nova (A)	Dec-02	6	1	CA	A2	33.8	1
PT156	Idanha-a-Nova	Dec-02	6	1	CA	A2	34.3	1
PT157	Qta. Mourinhos, Idanha-a-Nova (A,D)	Dec-02	6	1	CA	A2	34.8	1
PT158	Qta. Mourinhos, Idanha-a-Nova (A,D)	Dec-02	6	1	CA	A2	33.3	1
PT159	Ladoeiro, Idanha-a-Nova	Dec-02	6	1	CA	A2	39.7	1
PT160	Monforte da Beira, Castelo Branco	Dec-02	6	2	CA	A2	36.0	1
PT161	Alfrívida, Vila Velha de Ródão (A)	Dec-02	6	3	CA	A2	35.5	1
PT162	Alfrívida, Vila Velha de Ródão (A)	Dec-02	6	3	CA	A2	34.8	1
PT163	Sarnadas, Vila Velha de Ródão (A)	Dec-02	6	3	CA	A2	32.7	1
PT164	Sarnadas, Vila Velha de Ródão (A)	Dec-02	6	3	CA	A2	33.0	1

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TABLE 1—*Continued*

Reference[a]	Location[b]	Collection date (mo-yr)	Grove, olive trees[c]	Topography[d]	Species[e]	*C. acutatum* groups	Colony diam after 5 days on PDA 25°C (mm)	Growth on PDA with benomyl (2 mg/dm^{-3}), 5 days at 25°C[f]
PT165	Carapetosa, Vila Velha de Ródão (A,B)	Dec-02	4	3	CA	A2	36.3	1
PT166	Carapetosa, Vila Velha de Ródão (A,B)	Dec-02	4	3	CA	A2	36.2	1
PT167	Vila Viçosa (A,E)	Dec-02	5	2	CA	A2	31.3	1
PT168	Vila Viçosa (A,E)	Dec-02	5	2	CA	A2	35.7	1
PT169	Qta. Cavadinha, Sabrosa (F)	Nov-02	4	4	CA	A4	30.5	1
PT170	Dois Portos, Torres Vedras (G)	Dec-02	3	2	CA	A3	30.2	1
PT171	Asseiceira, Rio Maior (H)	Dec-02	6	2	CA	A4	35.5	1
PT172	Estanganhola, Rio Maior	Dec-02	5	2	CA	A2	30.5	1
PT173	Alcanena (I)	Dec-02	5	3	CA	A2	34.0	1
PT174	Torres Novas	Dec-02	5	1	CA	A2	34.5	1
PT177	Anceriz, Arganil (A)	Dec-02	4	4	CA	A2	30.9	1
PT178	Anceriz, Arganil (A)	Dec-02	4	4	CA	A2	29.8	1
PT179	Anceriz, Arganil (A)	Dec-02	4	4	CA	A2	33.2	1
PT180	Anceriz, Arganil (A)	Dec-02	4	4	CA	A2	29.8	1
PT181	Ladoeiro, Idanha-a-Nova	Dec-02	4	1	CA	A2	36.2	1
PT182	Ervidel, Aljustrel	Nov-02	3	1	CA	A2	34.3	1
PT183	Idanha-a-Nova	Dec-02	6	1	CA	A2	33.0	1
PT184	Monforte da Beira, Castelo Branco	Dec-02	6	2	CA	A2	36.8	1
PT186	Monte Barrão, Alter do Chão	May-03	6	1	CA	A2	34.0	1
PT187	Monte Barrão, Alter do Chão	May-03	6	1	CA	A2	33.2	1
PT201	Vila Viçosa (E)	Oct-03	5	2	CA	A2	32.5	1
PT202	Qta. Mourinhos, Idanha-a-Nova (D)	Oct-03	6	1	CA	A2	35.0	1
PT203	Qta. Vale de Cardas, Idanha-a-Nova	Oct-03	6	3	CA	A2	41.2	1
PT204	S. Miguel de Acha, Idanha-a-Nova	Oct-03	2	2	CA	A2	30.2	1
PT205	Elvas	Nov-03	6	2	CA	A2	33.5	1
VM206	Patacão, Faro	Nov-03	2	1	CG		46.8	0
VM207	Alcácer do Sal	Nov-03	2	2	CA	A2	33.5	1
VM208	Luz, Tavira	Nov-03	5	1	CA	A2	32.5	1
PT210	Minde, Alcanena	Nov-03	2	2	CA	A2	34.2	1
PT211	Moitas-Venda, Alcanena	Nov-03	3	4	CA	A2	31.7	1
PT212	Alcanena (I)	Nov-03	5	2	CA	A2	35.0	1
PT213	Luz, Tavira	Nov-03	5	1	CA	A2	42.8	1
PT214	Reguengo do Fetal, Batalha	Nov-03	3	4	CA	A2	32.0	1
PT215	Abrigada, Alenquer	Nov-03	2	2	CA	A2	35.7	1
PT216	Amiais de Cima, Santarém	Nov-03	5	2	CA	A2	36.2	1
PT217	Elvas (C)	Nov-03	6	2	CA	A2	30.7	1
PT218	Qta. Vale de Cardas, Idanha-a-Nova	Oct-03	6	3	CA	A2	34.8	1
PT219	Caria, Belmonte	Dec-03	6	1	CA	A2	33.7	1
PR220	Batoco, Tondela	Dec-03	1	3	CG		53.3	0
PT222	Aldeia da Ribeira, Santarém	Nov-03	5	2	CA	A2	32.2	1
PT223	S. João da Ribeira, Rio Maior (H)	Nov-03	5	2	CA	A2	32.8	1
PT224	Dois Portos, Torres Vedras (G)	Nov-03	3	3	CA	A2	34.0	1
PT225	Ladeira, Ourém	Nov-03	2	2	CA	A2	35.7	1
PT226	Correias, Rio Maior (H)	Nov-03	5	1	CA	A2	33.0	1
PT227	V.N. Cacela, V.R. Sto. António	Nov-03	5	1	CA	A5	35.0	1
PT228	Gáfete, Crato	Oct-03	4	1	CA	A2	31.2	1
PT230	Sta. Comba da Vilariça, Vila Flor	Dec-03	5	4	CA	A2	37.7	1
PT231	Bouça, Mirandela	Dec-03	6	1	CA	A4	32.8	1
PT232	Valpaços	Dec-03	4	2	CA	A4	26.2	1
PT233	Carapetosa, Vila Velha de Ródão (B)	Dec-03	5	2	CA	A2	37.7	1
PT234	Perdigão, Vila Velha de Ródão	Dec-03	6	4	CA	A2	36.5	1
PT235	Várzea dos Cavaleiros, Sertã	Dec-03	5	3	CA	A2	33.0	1
PT236	Aveleira, Sertã	Dec-03	5	3	CA	A2	34.0	1
PT237	Pedrógão Grande	Dec-03	5	3	CA	A2	33.0	1
PT238	Graça, Pedrógão Grande	Dec-03	5	2	CA	A2	35.5	1
PT239	Chão de Couce, Ansião	Dec-03	4	2	CA	A2	35.8	1
PT241	Pedrógão Pequeno, Sertã	Dec-03	5	2	CA	A2	35.7	1
PT242	Casais, Proença-a-Nova	Dec-03	5	3	CA	A2	37.8	1
PT247	Lisbon	Dec-03	6	3	CA	A4	37.7	1
PT248	Valpaços	Dec-03	4	2	CA	A4	29.0	1
PT249	Dois Portos, Torres Vedras (G)	Nov-03	3	3	CA	A3	34.5	1
PT250	Torre de D. Chama, Mirandela	Dec-03	5	3	CA	A6	27.8	1
PT251	Valpaços	Dec-03	6	2	CA	A2	31.0	1
PT253	Dois Portos, Torres Vedras (G)	Nov-03	2	2	CA	A2	34.5	1
PT254	Qta. Cavadinha, Sabrosa (F)	Dec-03	4	4	CA	A4	36.8	1

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TABLE 1—*Continued*

Reference[a]	Location[b]	Collection date (mo-yr)	Grove, olive trees[c]	Topography[d]	Species[e]	*C. acutatum* groups	Colony diam after 5 days on PDA 25°C (mm)	Growth on PDA with benomyl (2 mg/dm^{-3}), 5 days at 25°C[f]
CBS193.32	Italy	1932			CA	A4	32.3	1
M3	Serbia and Montenegro	2003			CA	A4	29.0	1
M4	Serbia and Montenegro	2003			CA	A4	33.7	1

[a] EMBL accession numbers for ITS and *tub2* sequences respectively, for each isolate: AJ749681/AJ748605 (isolate PT108); AJ749682/AJ748606 (PT111); AJ749683/AJ748607 (PT135); AJ749684/AJ748608 (PT166); AJ749685/AJ748609 (PT169); AJ749686/AJ748610 (PT170); AJ749687/AJ748611 (PT186); AJ749691/AJ748615 (PT201); AJ749692/AJ748616 (VM206); AJ749693/AJ748617 (PR220); AJ749694/AJ748618 (PT227); AJ749695/AJ748619 (PT231); AJ749696/AJ748620 (PT232); AJ749697/AJ748621 (PT247); AJ749698/AJ748622 (PT248); AJ749699/AJ748623 (PT249); AJ749700/AJ748624 (PT250); AJ748625 (PT254, *tub2* only); AJ749688/AJ748612 (CBS193.32); AJ749689/AJ748613 (M3); AJ749690/AJ748614 (M4); AJ749670/AJ748626 (CA302a); AJ749671/AJ748627 (PD443); AJ749672/AJ748628 (CA473); AJ749673/AJ748630 (CA287); AJ748632 (PD85/694, *tub2* only); AJ749676/AJ748633 (CA455); AJ749677/AJ748634 (CA318); AJ749678/AJ748635 (PD89/582); and AJ749679/AJ748636 (PD88/673).

[b] For each location, A indicates isolates that were obtained from the same fruit to monitor the existence of mixed populations; labels B to H indicate isolates obtained in different years from geographically related locations to monitor population shifts.

[c] Grove: 1, wild olive trees (*Olea europaea* var. *sylvestris*); 2, isolated olive trees in urban areas; 3, olive trees by roadsides; 4, olive trees within another crop; 5, old grove; 6, young or mature grove.

[d] Topography: 1, plain; 2, undulated; 3, hilly; 4, mountainous.

[e] Species: CA, *C. acutatum;* CG, *C. gloeosporioides.*

[f] 0, no growth (100% inhibition); 1, partial growth (27 to 67% inhibition).

though considerable diversity was recorded among the 128 *C. acutatum* isolates, most of these clustered with previously characterized reference *C. acutatum* isolates and represented molecular groups A2 to A5; none clustered with group A1 (Table 1 and Fig. 2 and 3). For example, 114 isolates collected from different parts of Portugal clustered with reference isolates CA397 and PD85/694, representing group A2. These 114 isolates, represented by isolates PT135 and PT201 (Fig. 2 and 3), showed high degree of relatedness among them with over 91% Dice similarity coefficient but exhibited only 60 to 80% similarity to the reference isolates CA397 and PD85/694. Isolates PT170 and PT249 (from Torres Vedras) clustered with reference isolates CR46 (grapevine) and CA473 (tulip tree), representing group A3. Isolates PT169, PT171, PT231, PT232, PT247, PT248, and PT254 (from Rio Maior, Lisbon, and different locations in the Trás-os-Montes region) as well as CBS193.32 (collected in Italy in 1932) and M3 and M4 (from Serbia and Montenegro), clustered together with reference isolates TN47 (medlar) and NI90 (strawberry), representing group A4. Isolate PT169 (Fig. 2 and 3) represented isolates PT171, PT232, PT248, and PT254, and CBS193.32 represented isolates M3, M4, PT231, and PT247. Isolate PT227 (from Vila Real de Santo António) was the single olive isolate grouping with PD443, representing group A5. Isolate PT250 (from Mirandela), which was distinct from all other olive anthracnose *C. acutatum* isolates, as well as the various reference *C. acutatum* isolates, was assigned to A6. Within the *C. gloeosporioides* cluster, isolate PT111 (from Vila Velha de Ródão) represented isolates VM206 and PR220 (from Faro and Tondela, respectively) (Fig. 2 and 3), with no differences found among the three. All the groups referred to are supported by bootstrap values of >80%.

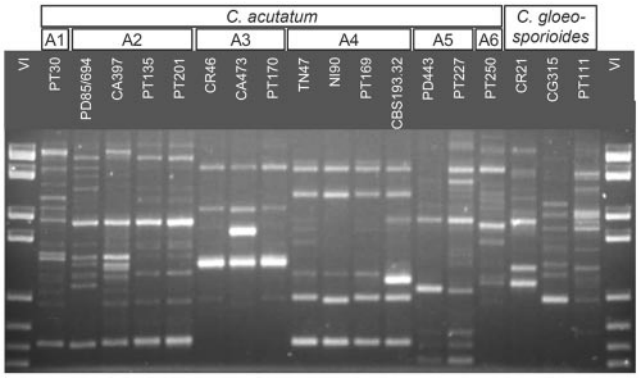

FIG. 2. Arbitrarily primed PCR profiles obtained with primer MR for a representative set of *Colletotrichum* spp. isolates from olive along with reference *C. acutatum* and *C. gloeosporioides* isolates. PT30 (A1), PD85/694 and CA397 (A2), CR46 and CA473 (A3), TN47 and NI90 (A4), and PD443 (A5) are reference isolates for *C. acutatum* molecular groups shown. CR21 and CG315 are reference isolates for *C. gloeosporioides*. VI is a molecular marker (Type VI; Roche Diagnostics, Mannheim, Germany). Full details of the isolates and the *C. acutatum* molecular groups to which all the analyzed isolates belong are shown in Table 1.

Nucleotide sequence analysis. Nucleotide sequences of the rRNA gene-ITS region and a variable region of the *tub2* gene determined for a set of 19 to 20 representative isolates selected according to the molecular groups revealed by AP-PCR and analyzed along with a number of reference isolates showed comparable tree topologies (Fig. 4 and 5). Both the rRNA gene-ITS and the *tub2* datasets analyzed according to the Kimura-2P coefficient and unweighted-pair group method with arithmetic average (UPGMA) clustering clearly revealed that olive *Colletotrichum* isolates PT111, VM206, and PR220 clustered together with *C. gloeosporioides* reference isolates, while the remaining isolates clustered together with *C. acutatum* reference isolates with 100% bootstrap values in each case (Fig. 4 and 5). Diversity between the *C. acutatum* and *C. gloeosporioides* isolates ranged from 9.5 to 11.0% with ITS and 17.7 to 23.4% with *tub2*. The olive *C. acutatum* isolates PT108, PT135, PT166, and PT201 clustered in group A2. The ITS sequence of these isolates was identical and showed only 0.2% difference from AF081292, representing Spanish olive anthracnose isolates (21). Overall diversity within group A2 was higher, with 0.6% for ITS and 2.58% for *tub2*. Isolates PT170 and PT249 clustered into A3 and isolates CBS193.32, M3, M4, PT169, PT231, PT232, PT247, PT248, and PT254 grouped into

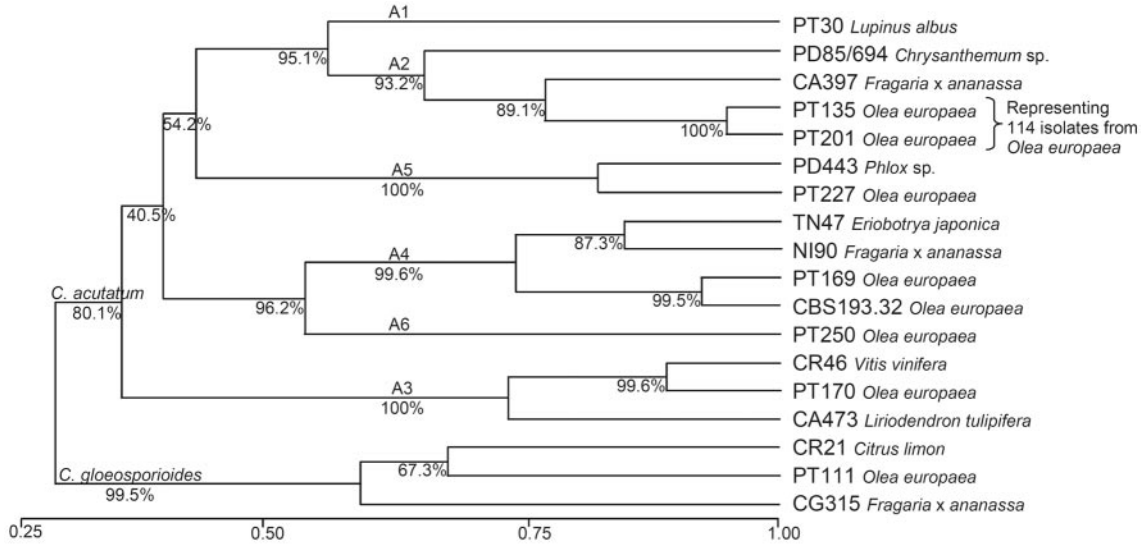

FIG. 3. Dendrogram showing the diversity and relationships among the olive anthracnose *Colletotrichum* spp. isolates based on cluster analysis (UPGMA) of a similarity matrix (Dice) generated from arbitrarily primed PCR profile data with seven different primers. A total of 2,000 bootstraps were used. Cophenetic correlation coefficient $r = 0.95787$. Isolates PT135 and PT201 represent 114 olive anthracnose isolates clustering in group A2. Details of the previously characterized reference isolates (34) used for *C. acutatum* molecular groups A1 to A5 and *C. gloeosporioides* reference isolates are described in the legend to Fig. 2. Full details of the isolates and the *C. acutatum* molecular groups to which all the analyzed isolates belong are shown in Table 1.

A4. Isolate PT227 grouped in A5 together with ITS sequences AF411700 and AF411701, representing, respectively, the species holotype and paratype (36), and isolate PT250 clustered in group A6 together with ITS sequence AF411704 (*C. acutatum* on *Rhododendron* sp.) (36). No olive *Colletotrichum* spp. isolates clustered together with group A1 (Fig. 4 and 5). Among the *C. acutatum* isolates, minimum intergroup divergence was 0.8% for ITS (between A5 and A2 or A3) and 3.0% for *tub2* (between A1 and A2). Maximum intergroup divergence was 4.47% for ITS (between A1 and A3 or A4) and 4.89% for *tub2* (between A3 and A4).

Cultural and morphological characteristics. Measurements of the mycelial growth on PDA (Table 1) distinguished two types of isolates. The first type (*C. acutatum*) contained 128 of the 131 tested *Colletotrichum* spp. isolates from olive, including CBS 193.32 from Italy, M3 and M4 from Serbia and Montenegro isolates, and *C. acutatum* reference isolates, with a radial growth of 18.0 to 45.0 mm. The second type (*C. gloeosporioides*) included olive *Colletotrichum* isolates PT111, VM206, and PR220 and *C. gloeosporioides* reference isolates, with radial growth of 52.0 to 62.0 mm (significantly higher than that of *C. acutatum* isolates). These two types of isolates could also be distinguished based on mycelial growth on PDA containing 2 mg $\cdot$ dm^{-3} benomyl, where the *C. gloeosporioides* isolates exhibited 100% inhibition of mycelial growth, and *C. acutatum* isolates showed only partial (27 to 67%) inhibition. Within the olive *C. acutatum* isolates, colony characteristics differentiated a number of groups that, in most cases, corresponded to the groups recorded by molecular characterization: the vast majority of isolates showed white to beige colonies with greyish specks; occasionally, the lower surface was olive green (isolates VM207, PT213, and PT235) or yellow (isolate PT210), corresponding to *C. acutatum* group A2 (Table 1); 10 isolates had beige to grey colonies, but the center of the colony was covered with abundant orange masses of conidia, corresponding to group A4; colonies of A3 isolates (PT170 and PT249) were light pink to carmine/flesh-colored; colony of the A5 isolate (PT227) was strong purple to red; the A6 isolate (PT250) had a grey colony (data not shown).

Conidia size and shape assessment revealed high variability within each isolate and little statistically significant difference among different isolates. Conidial length was 7.7 to 12.7 μm and width was 2.8 to 4.5 μm for conidia originating from colonies grown on PDA, while length and width of conidia from SNA colonies were, respectively, 12.4 to 15.0 μm and 3.2 to 4.5 μm. No clear grouping of the *Colletotrichum* spp. isolates from olive was observed, apart from isolates PT111, VM206, and PR220, which along with the *C. gloeosporioides* reference isolates had mainly round-ended conidia from SNA cultures; isolate PT227 produced 100% acute-ended conidia on both PDA and SNA. Both the dimensions and shape of conidia recorded from SNA cultures were more uniform than from those from PDA cultures (data not shown). As an example, the average standard deviation for conidial length for a selected set of isolates was 1.74 μm on PDA and 0.96 μm on SNA.

Pathogenicity and disease incidence. A selection of olive *C. acutatum* and *C. gloeosporioides* isolates generally representing the molecular and phenotypic diversity observed above were tested for their pathogenicity on olive and other hosts. On olive fruits, abundant sporulation and frequently intense production of mycelium were observed with all isolates tested, including the reference isolates CR20 and CR21. Based on the reactions with fruits from 11 different olive cultivars, some differences in virulence were recorded among olive *Colletotrichum* spp. isolates. For example, among *C. acutatum*, isolate PT135 from group A2 was more virulent (1.753 ± 0.783 on a symptom scale of 0 to 3; values are averages $\pm$ standard deviation of symptom rating obtained in sets of 10 fruits from 11 different cultivars)

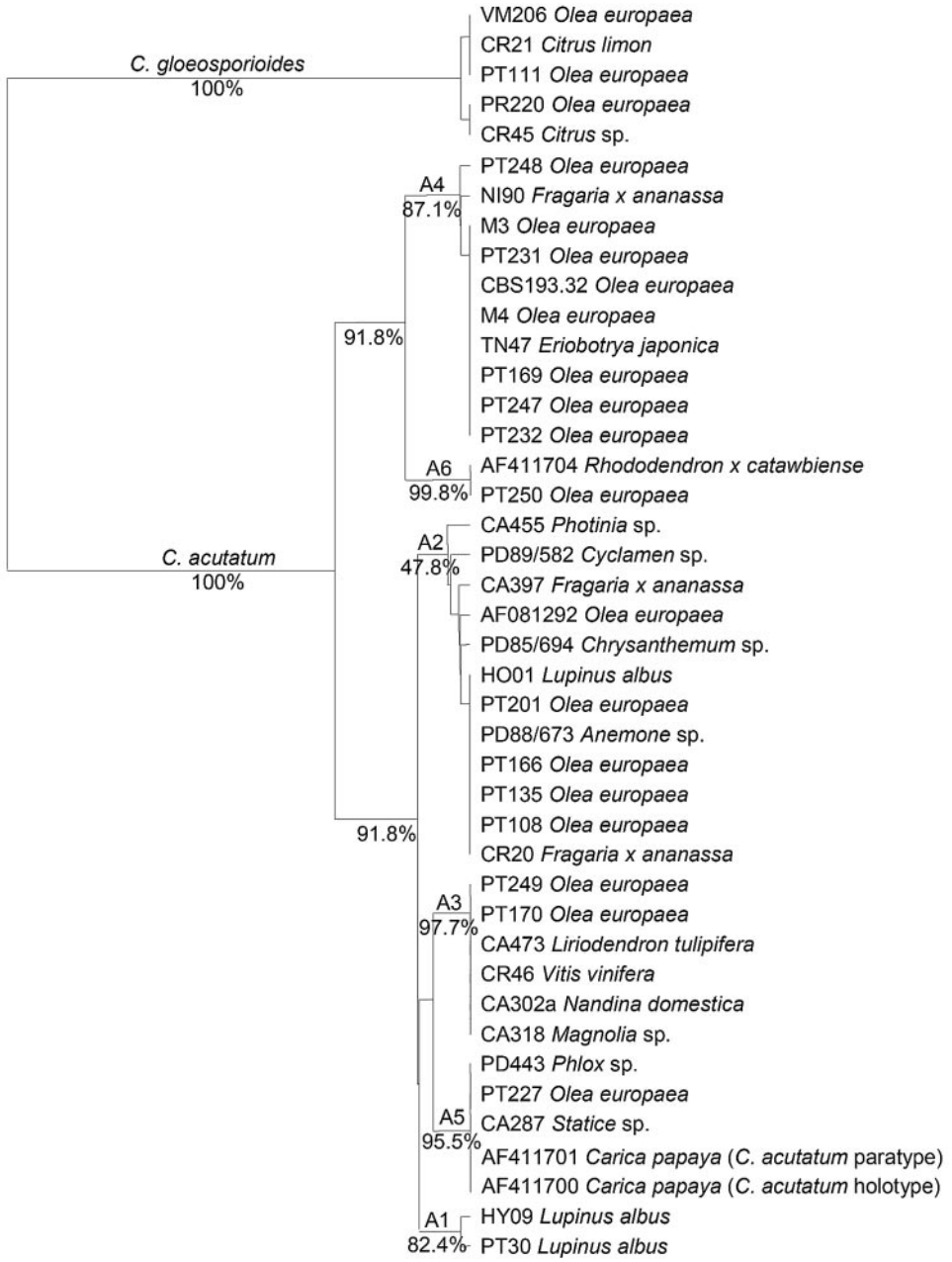

FIG. 4. UPGMA consensus dendrogram depicting relationships among *Colletotrichum* spp. isolates from olive with *C. acutatum* and *C. gloeosporioides* reference isolates based on rRNA gene-ITS sequences. Sequences AF081292, AF411700, AF411701, and AF411704 were retrieved from nucleotide sequences databases. A total of 2,000 bootstrap data sets and Kimura-2P model distance matrices were used. Bootstrap values are only shown for key nodes. Full details of the isolates are shown in Table 1. *C. acutatum* and *C. gloeosporioides* isolates from hosts other than olive have been used as reference isolates, and these isolates have been previously characterized using various molecular markers (31, 34). A1 to A6, *C. acutatum* molecular groups.

than isolates PT170 from group A3 (1.154 ± 0.718) and PT169 from group A4 (1.274 ± 0.643). *C. gloeosporioides* isolate PT111 showed a virulence rating of 1.874 ± 0.430.

Further, olive *C. acutatum* isolates PT107, PT109, PT110, PT115, PT130, PT135, PT140, PT212 (group A2), PT170 and PT249 (group A3), PT169 and PT231 (group A4), PT227 (group A5), and CR20, as well as olive *C. gloeosporioides* isolates PT111 and VM206 and CR21 inoculated on strawberry fruits, produced sunken necrotic lesions with orange masses of conidia and/or abundant aerial grey-whitish mycelia. No significant difference in virulence was recorded among the different isolates tested. Similarly, when strawberry and lupin plants were inoculated with the same set of isolates, no major differences in virulence were recorded and necrotic lesions on petioles, stems/runners, cotyledons, and leaves were produced by all isolates, and the pathogen was reisolated from diseased tissues (data not shown).

Olive anthracnose incidence was higher during the autumns

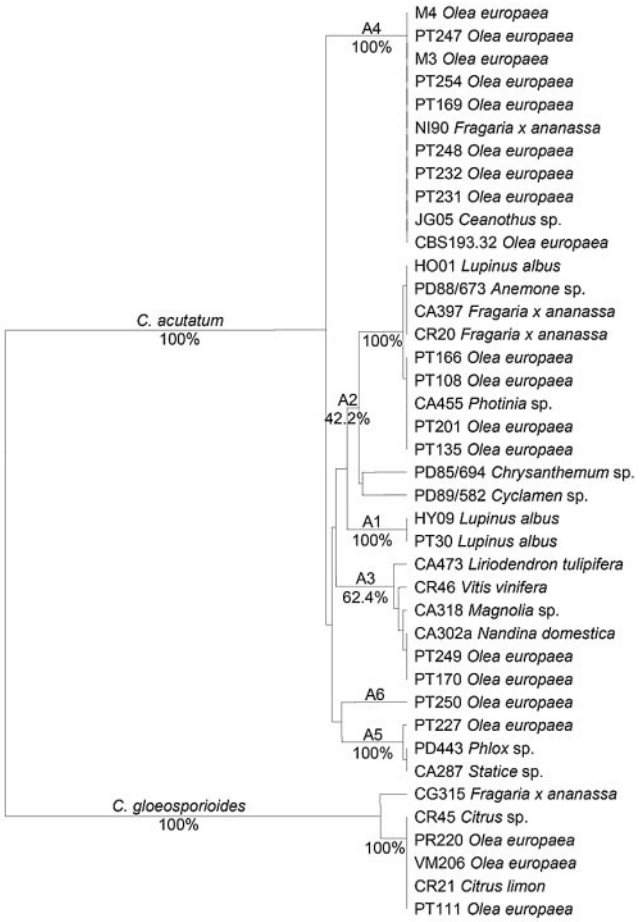

FIG. 5. UPGMA consensus dendrogram depicting relationships among *Colletotrichum* spp. isolates from olive, along with *C. acutatum* and *C. gloeosporioides* reference isolates, based on the nucleotide sequences of a variable region of the β-tubulin 2 gene. A total of 2,000 bootstrap data sets and Kimura-2P model distance matrices were used; bootstrap values are only shown for key nodes; full details of the isolates are shown in Table 1. *C. acutatum* and *C. gloeosporioides* isolates from hosts other than olive have been used as reference isolates, and these isolates have been previously characterized with various molecular markers (34). A1 to A6, *C. acutatum* molecular groups.

of 2001 (disease recorded in 20 of 25 groves surveyed) and 2002 (23 of 34 groves) compared to 2003 (34 of 110 groves). However, among the rest of the groves surveyed in 2003, at least 15 samples developed symptoms upon incubation under high humidity in the laboratory (only 31% of groves had symptoms, but inoculum was present in at least 45% of the groves) enabling pathogen isolation. The proportion of groves exhibiting anthracnose symptoms varied considerably across Portugal during the autumn of 2003; in general, disease incidence was much lower in Trás-os-Montes (9%) than in the rest of the country (42%). Anthracnose incidence also varied during the maturation season, ranging from 31% (including groves where the pathogen was present but symptoms were not visible) in late October to 93% in late November, excluding the Trás-os-Montes area where the incidence of disease in general was lower. The majority of the isolates from these surveys belonged to *C. acutatum* group A2, while most others belonged to *C. acutatum* groups A3 to A6; only three isolates belonged to *C. gloeosporioides* (Table 1 and Fig. 1). Further, in a particular location (Alter do Chão) during spring 2003, 4-year-old olive plants of cultivar Maçanilha exhibited elongated cankers on branches from which isolates PT186 and PT187 belonging to *C. acutatum* group A2 were isolated (Table 1).

Pathogen detection and diagnosis of *C. acutatum* molecular groups by PCR. Based on the nucleotide sequence data generated for the variable region of *tub2* gene (a ca. 550-bp fragment, including three different introns), PCR primers specific for *C. acutatum* (TBCA) and *C. gloeosporioides* (TBCG) were designed. Species-specific PCR tests using the *tub2* primers for *C. acutatum* (TB5 and TBCA) and *C. gloeosporioides* (TB5 and TBCG) identified 128 of the 131 *Colletotrichum* spp. isolates from olive as *C. acutatum*, along with reference isolates CMG12, PT30, PD85/694, CA397, CA473, NI90, and PD443. Isolates PT111, VM206, and PR220 were identified as *C. gloeosporioides*, along with reference isolates CR21 and CG315 (Fig. 6). Comparative analysis of these samples with the previously established rRNA gene-ITS based specific PCR for *C. acutatum* and *C. gloeosporioides* fully confirmed these results (Fig. 6). A clear PCR product was obtained when *C. acutatum*-specific primers (either for *tub2* or ITS) were used with DNA extracted directly from olive fruits showing anthracnose symptoms (the DNA extraction was carried out according to the protocol by Pasqualone et al.) (27). On the contrary, DNA extracted from healthy fruits or healthy leaves yielded no pathogen-specific PCR fragment, either with *C. acutatum*- or with *C. gloeosporioides*-specific primers. These DNA samples were clearly PCR amplifiable as tested with conserved primers, and the nucleotide sequence for rRNA gene-ITS region of olive cultivar Cobrançosa was consequently obtained and deposited in EMBL (accession no. AJ585193).

For the diagnosis of molecular groups within *C. acutatum*, *tub2* nucleotide sequences were analyzed with MapDraw 5.03 (DNAstar Inc., Madison, WI), and a set of restriction enzymes was selected, enabling the production of unique profiles for each of the *C. acutatum* molecular groups A1 to A6. These enzymes were applicable to the 328- to 330-bp *C. acutatum*-specific fragment obtained with primers TB5 and TBCA, as well as to the 549- to 551-bp fragment obtained with the conserved primers TB5 and TB6 (34). A maximum of three separate restriction reactions was required to identify the various groups as follows. (i) Digestion of either TB5 and TBCA or TB5 and TB6 amplified fragments of *tub2* with StyI produced unique profiles for isolates of *C. acutatum* groups A2, A3, and A5 (Fig. 7), but A1, A4, and A6 were indistinguishable. (ii) The use of NlaIII allowed the identification of A1. (iii) Digestion with SacI distinguished A6, identifying the rest of the isolates as A4 (Fig. 7). Nevertheless, to test whether a *C. acutatum* isolate belonged to a particular group, identification based on a single enzyme digestion is achievable using StyI for groups A2, A3, and A5 or SacI for A6. Two separate restriction digestions with StyI and NlaIII were required for group A1; similarly, RsaI and NlaIII digestions were required for A4.

DISCUSSION

Molecular and phenotypic characterization of 131 *Colletotrichum* spp. isolates associated with olive anthracnose and

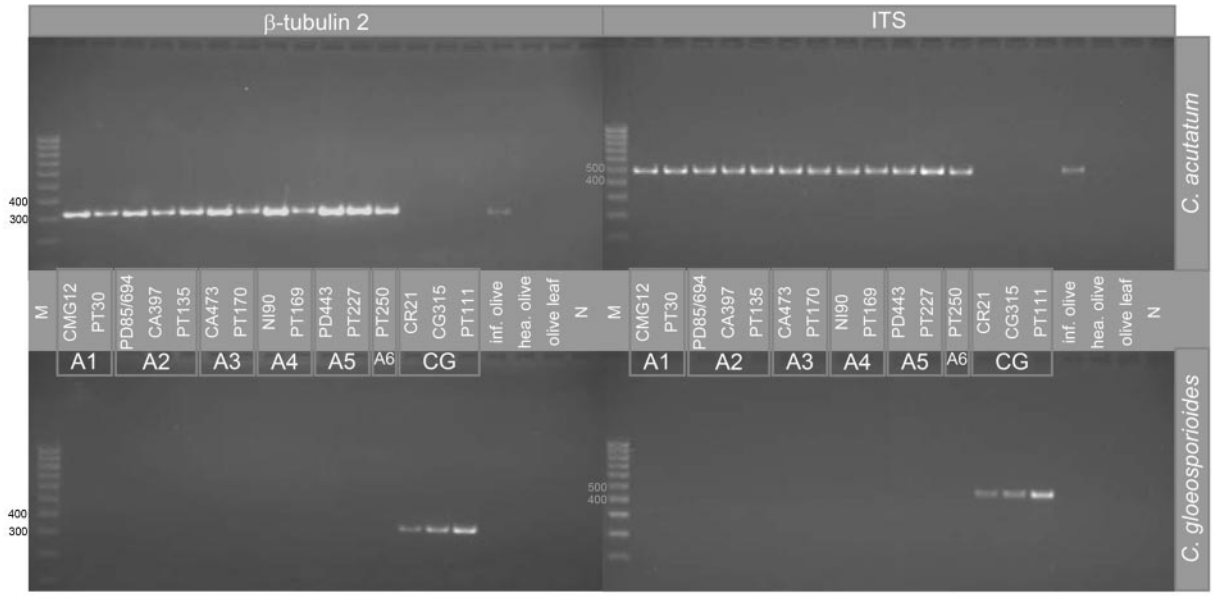

FIG. 6. Agarose gel electrophoresis of PCR products obtained with DNA of some of the *Colletotrichum* spp. isolates from olive and with DNA extracted from infected olive fruits using β-tubulin 2 primers and rRNA gene-ITS primers specific for *C. acutatum* and *C. gloeosporioides.* (Top left) *C. acutatum*-specific products generated with β-tubulin 2 primers TB5 and TBCA from fungal cultures and infected olive fruit; (bottom left) *C. gloeosporioides*-specific products generated with β-tubulin 2 primers TB5 and TBCG from fungal cultures; (top right) *C. acutatum*-specific products generated with rRNA gene-ITS primers CaInt2 and ITS4 (32) from corresponding samples on left; (bottom right) *C. gloeosporioides*-specific products generated with rRNA gene-ITS primers CgInt and ITS4 (23) from corresponding samples on left. DNA from healthy olive fruits and olive leaves and water were used as controls. M, molecular marker (100-bp Low; Invitrogen, Paisley, United Kingdom); CG, *C. gloeosporioides*; A1 to A6, *C. acutatum* molecular groups.

comparative analysis with a range of reference *C. acutatum* and *C. gloeosporioides* isolates identified 128 isolates as *C. acutatum* and only 3 isolates as *C. gloeosporioides.* AP-PCR markers and rRNA gene-ITS and *tub2* nucleotide sequence data strongly supported these results. Cultural characteristics such as colony radial growth and (particularly) benomyl sensitivity also revealed two distinct types of isolates corresponding to *C. acutatum* and *C. gloeosporioides*, as has been observed with previous investigations of various hosts (1, 19, 34). On the contrary, conidial morphology was less informative than in previous studies (6, 33), although conidia were also assessed from SNA medium, recognized as producing less conidial variability (25). However, application of species-specific PCR using the *tub2*-based primers developed for *C. acutatum* and *C. gloeosporioides* in the current study, as well as the previously available primers based on rRNA gene-ITS (23, 32), provided rapid and reliable diagnosis of isolates belonging to *C. acutatum* and *C. gloeosporioides* from olives. Conflicting reports exist in the literature on the causal agent(s) of olive anthracnose in different geographic locations (3, 5, 17, 20, 21, 24), and the current study has clearly established *C. acutatum* as the dominant pathogen (>97%) with very limited occurrence of *C. gloeosporioides* (<3%). This is the first report on the existence of diverse molecular groups among *C. acutatum* populations associated with olive anthracnose. AP-PCR markers, as well as the ITS and *tub2* nucleotide sequences, consistently divided the *C. acutatum* populations into five molecular groups, which mostly correlated with various morphological groups based on different colony characteristics. Interestingly, at least three of these groups correspond to previously described *C. acutatum* groups A2, A3, and A4 from other hosts (34), while A5 and A6 are newly described groups. On the other hand, no olive anthracnose isolates clustered into *C. acutatum* group A1, which comprises the vast majority of isolates causing lupin anthracnose (34). Thus in all, *C. acutatum* populations associated with a wide range of hosts appear to fit into at least six molecular groups, which could be related to various *C. acutatum* sensu lato groups described based on morphology and randomly amplified polymorphic DNA analyses (16) and mitochondrial DNA-restriction fragment length polymorphism and sequence variations in introns of two different genes (13).

The vast majority of *C. acutatum* olive anthracnose isolates displaying >91% similarity belonged to group A2 (89%), which included isolates collected from major olive-producing areas of Alentejo (100% of isolates were A2), Ribatejo (94% were A2), and Beira Baixa (97% were A2) in Portugal. Groups A3, A4, A5, and A6 comprised the remaining 11% *C. acutatum* isolates and were mostly restricted in their occurrence, except A4, which is predominant in the Trás-os-Montes region (where 5 out of 8 isolates were A4, while only 2 out of 120 isolates from the rest of Portugal were A4). This represents a different scenario than with *C. acutatum* populations from other hosts. For example, two distinct clonal subpopulations were found on almonds in California (8), a clonal group and a variable group were found on strawberries in Europe and America (7), and one homogenous group mainly represented a global collection from lupins (34). Further, preliminary comparative analysis of the olive anthracnose isolates from Portugal with the limited information available elsewhere (3, 21, 38) suggests that *C.*

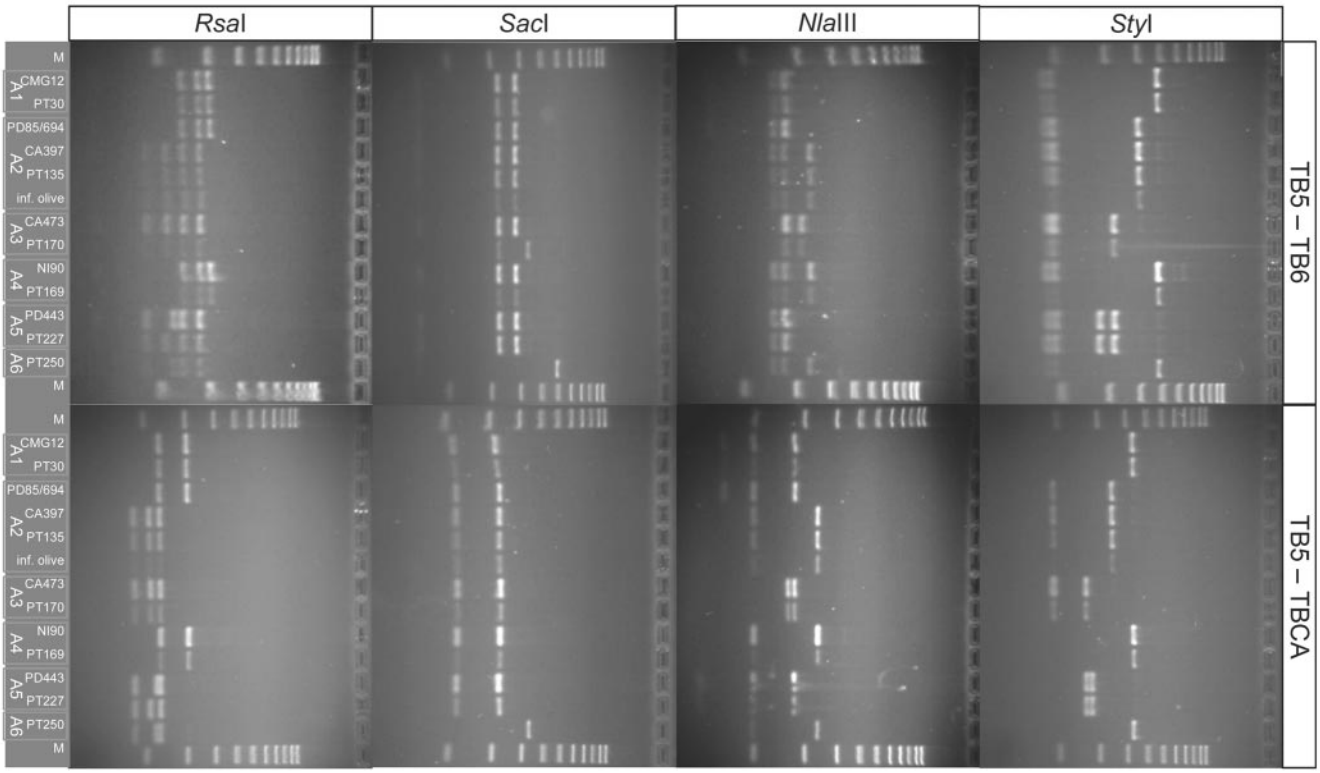

FIG. 7. Restriction profiles of β-tubulin 2 fragments amplified by conserved primers TB5 and TB6 as well as *Colletotrichum acutatum*-specific primers TB5 and TBCA obtained with enzymes StyI, NlaIII, SacI, and RsaI enabling the distinction of *C. acutatum* groups A1 to A6 (groups distinguishable with each enzyme are shown next to enzyme name). Isolates representing each group were selected to cover the genetic diversity determined within these groups based on nucleotide sequence analyses (Fig. 4 and 5). PCR product directly amplified from an infected olive sample and digested with various enzymes is also included (inf. olive). M, molecular marker (100-bp Low; Invitrogen, Paisley, United Kingdom). A limited number of isolates yielded atypical profiles with some enzymes that were not used for distinguishing the corresponding groups (for example, isolate PD85/694 identified as A2 with StyI yielded an A1-like profile with NlaIII; isolate CA473 identified as A3 with StyI yielded a non-A3 profile with SacI for the TB5 and TB6 fragment) and did not affect the group identifications.

acutatum groups A2 and A4 are likely to be the key pathogens in other countries.

Within Portugal, *C. acutatum* isolates PT170 and PT249 (A3) from Torres Vedras, PT227 (A5) from Vila Real de Santo António, and PT247 (A4) from Lisbon, as well as *C. gloeosporioides* isolates VM206 from Faro and PR220 from Tondela, were obtained from areas where olive cultivation is less important/marginal. These could represent cross-infection events or recently adapted pathogens, with *Colletotrichum* pathogens from other hosts causing anthracnose symptoms on olive, as has been observed by Freeman et al. (12) among *C. acutatum* populations from anemone and strawberry. Interestingly, during the autumns of 2001 and 2002, when meteorological conditions were particularly favorable and anthracnose incidence was high (68 to 80% of groves had symptoms), *C. acutatum* group A2 was vastly dominant (95% of isolates obtained). In the autumn of 2003, with less favorable conditions and lower incidence (31% of groves had symptoms), the proportion of isolates not belonging to *C. acutatum* group A2 was much higher (21%). A different scenario occurs in Trás-os-Montes, where olive anthracnose used to be very rare (R. Sismeiro, personal communication). The 9% incidence recorded in 2003 surveys reflects recent spread of the disease, and the main pathogen found was *C. acutatum* group A4, along with isolates from A2 and A6. Group A4 included previously characterized *C. acutatum* isolates from medlar, strawberry, and *Ceanothus* sp., while group A3 included isolates from grapevine and several ornamentals. Interestingly, *C. acutatum* isolates belonging to groups A3 and A4 have recently been isolated from grapevine and medlar, which are common in Portugal, the latter more frequently as a backyard fruit tree. Moreover, *C. acutatum* isolates belonging to different molecular groups showed differences in their virulence in olive, with A2 the dominant group tending to be more virulent than those from groups A3 and A4. Isolates clustering in *C. acutatum* group A2 are frequently isolated from strawberry (a common horticultural crop) and various ornamentals. A6 included some of the recently described *C. acutatum* isolates from *Rhododendron* spp. (36) and isolates from papaya, *Phlox* sp., and *Statice* sp. grouped in A5. This group included the *C. acutatum* holotype and paratype from papaya based on ITS sequences, from dry herbarium specimens obtained by Vinnere et al. (36). However, the fact that the type specimens are included in A5 may not necessarily mean that this group represents the early form of *C. acutatum*, as CBS193.32, originally identified from olives in 1932, is clearly shown in this study to be *C. acutatum*. This contradicts the view that *C. acutatum*, a taxon described by Simmonds in 1965 (30), spread recently due to the extensive

use of fungicides such as benomyl to which *C. acutatum* is moderately resistant (1, 14). Moreover, the acute-ended nature of conidia was one of the major criteria for the definition of *C. acutatum*, but it appears that only certain isolates clustering directly with the type specimen in *C. acutatum* group A5 (for example, isolate PT227) possessed 100% acute-ended conidia with grown on both PDA and SNA media. In general, considerable variation in conidial morphology was observed with the vast majority of *C. acutatum* isolates analyzed. This causes difficulties in reliably diagnosing the isolates where colony characteristics and molecular traits were compatible with *C. acutatum*, but conidia were not acute ended (34).

Cooccurrence of *C. acutatum* and *C. gloeosporioides* on a very limited scale was previously reported from Spain (21). In this study, despite analyzing more than one isolate from the same fruit from 17 different locations (41 isolates), these two pathogens were not observed together. *C. gloeosporioides* represents <3.0% of the total number of olive anthracnose isolates studied currently, which is comparable to the 3.7% level recorded in Spain (22). However, the symptoms on olive fruits from which *C. gloeosporioides* was isolated were indistinguishable from those caused by *C. acutatum*; equally importantly, the *C. gloeosporioides* isolate tested was at least as virulent as the most virulent *C. acutatum* isolates on mature detached olive fruits. *C. gloeosporioides* is well recognized as a maturation or postharvest pathogen in a wide range of fruit crops such as coffee (35), citrus (40), and several tropical or subtropical fruits such as avocado, guava, papaya, mango, and passion fruit (28). Thus, the role and relative importance of *C. gloeosporioides* in olive anthracnose and cooccurrence with *C. acutatum* need to be addressed. Further, isolates PT169 and PT171 belonging to *C. acutatum* group A4 were initially identified from two different locations. Of these, more A4 isolates were obtained subsequently at Trás-os-Montes where PT169 was found, whereas no anthracnose was recorded at the location where PT171 was found, and isolates from surrounding sites belonged to *C. acutatum* A2. Further monitoring is essential to determine whether any of the smaller *C. acutatum* groups will become more widespread. Significantly, isolates PT186 and PT187 belonging to *C. acutatum* A2 were also found on branches of young olive plants in the Alentejo region, suggesting that the elongated cankers on the branches were caused by the fruit anthracnose pathogen. Necrosis of olive leaves and shoots caused by anthracnose pathogens *C. acutatum* and *C. gloeosporioides* either naturally or under controlled conditions has previously been reported, and infected leaves and shoots were suggested as the major inoculum sources for fruit anthracnose in autumn (5, 20). Our observations indicate that overwintering infected fruit mummies could also serve as inoculum sources. Thus, the olive anthracnose pathogen epidemiology needs to be further investigated, as *C. acutatum* has also been reported to exhibit epiphytic, endophytic, and nonpathogenic lifestyles on other crops (9, 18).

In this context, the *C. acutatum*- and *C. gloeosporioides*-specific primers TBCA and TBCG, respectively, developed in this work together with the conserved primer TB5 (34) amplified the *tub2* gene fragment specific for these pathogens with all samples tested reliably. This included isolates belonging to all six *C. acutatum* molecular groups. These primers, based on the single-copy *tub2* gene (26), constitute an alternative to the use of ITS-based specific primers (23, 32), as there is some concern over the potential for existence of divergent ITS copies within monosporic fungal cultures (15). Combined use of diagnostic PCR and restriction digestion of the amplicon allows the rapid and reliable diagnosis of *C. acutatum* molecular groups, for example, using TB5 and TBCA. Amplicons with conserved primers TB5 and TB6 are also useful as they are larger, yielding better resolution of the digested products. The proposed set of enzymes was chosen taking into consideration the maximum nucleotide sequence variation occurring within each *C. acutatum* group: for example, isolates PD85/694 and CA397 in A2 and CA473 and PT170 in A3. A protocol for DNA extraction from olive fruits (27), stems, and leaves was tested, enabling the extraction of PCR-amplifiable DNA and successful direct detection of *Colletotrichum* spp. and group identification using these primers. The *C. acutatum*- and *C. gloeosporioides*-specific primers based on *tub2*, which is known as a housekeeping gene, are also likely to be useful for RT-PCR-based quantification of viable pathogen propagules in asymptomatic and early infections.

This work has clearly demonstrated that diverse *C. acutatum* groups and a low level of *C. gloeosporioides* isolates are associated with olive anthracnose in Portugal. Further, there appears to be a degree of correlation between high disease incidence along with favorable environmental conditions and the widespread prevalence of *C. acutatum* group A2, as shown by the low incidence of disease and the frequency of group A4 in Trás-os-Montes and the sporadic occurrence of *C. acutatum* groups A3, A5, and A6 and *C. gloeosporioides* in marginal cropping locations in Portugal. Moreover, this work has shown the variation in the virulence of the olive anthracnose pathogen isolates, as well as their cross-infection potential, which has implications for both disease control and the host adaptability of pathogen populations. Further investigations are essential to understand the temporal and spatial dynamics of pathogen populations in relation to olive anthracnose spread and severity, as well as any interchange of pathogens among different hosts such as citrus, strawberry, medlar, almond, and peach in the Mediterranean region and other geographic locations like Australia, South Africa, and Chile. In these locations, there is growing interest in olive cultivation, and anthracnose has already been recorded (for example) in Australia (Barbara Smith, personal communication), China (20), and India (24). The diagnostic tools developed could be utilized in pathogen population analysis and epidemiology so that the knowledge and resources generated can be exploited for more efficient management of host resistance and improved disease control can be achieved.

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